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PROCESS FOR THE PREPARATION OF CLAVULANIC ACID

This invention relates to a process for preparation of clavulanic acid.

Clavulanic acid is the common name for (2R, 5R, Z) - 30(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid. Clavulanic acid and its alkali metal salts and esters are active as inhibitors of beta lactamase produced by some Gram positive as well as Gram negative micro-organisms. In addition to inhibition of beta lactamase, clavulanic acid and alkali metal salts thereof also have a synergistic action with penicillin and cephalosporin antibiotics. Clavulanic acid and its salts are used in pharmaceutical preparations to prevent the deactivation of beta lactam antibiotics. Commercial preparations contain potassium clavulanate in combination with amoxycillin trihydrate. Potassium clavulanate is more stable than the free acid or other salts.

Clavulanic acid is prepared by fermentation of micro-organisms such as strains of Streptomyces for example S.clavuligerus NRRL 3585, S.jumonjinensis NRRL 5741 and S.katsurahamanus IFO 13716 and Streptomyces sp.P6621 FERM P2804. The aqueous culture obtained after fermentation is purified and concentrated in accordance with conventional processes for example filtration and chromatographic purification as disclosed in GB 1508977, prior to extraction of the aqueous solution with an organic solvent to obtain a solution of impure clavulanic acid in the solvent.

WO95/23870 and WO96/28452 disclose improved commercial processes for purification of clavulanic acid and preparation of potassium clavulanate. Growth of an antibiotic producing micro-organism may include two phases; the growth phase during which biomass is produced and the subsequent stationary phase during which growth does not incur. Secondary metabolites such as antibiotics are usually produced during the stationary phase.

Fed batch fermentation processes are well known for

antibiotic production and have been preferred for production of clavulanic acid. Control and maintenance of desired levels of assimilable sources of nitrogen and carbon in the fermentation broth is well illustrated by Lee J S et al, Kor.Jour.Microbiol. 1978, Vol 15, No 1, p 21-29, which describes the improvement of the fermentation process for preparation of penicillin by control of addition of the assimilable nitrogen and carbon source according to the needs of microorganisms in the fermenter. Lilley G et al, J.Chem.Tech.Biotechnol. 1981, Vol 31, p 127-134 illustrates that the production of antibiotics by Streptomyces species can be controlled by changing of concentration of assimilable nitrogen and carbon source and source of phosphorus. For example, the production of thienamycin in the fermenter does not start until the concentration of phosphorus approaches zero. EP 82522 illustrates use of continuous or intermittent addition of the assimilable carbon source in fermentation of S.clavuligerus NRRL 3585. Regulation of the amount of ammonia is disclosed as WO96/18743. Continuous fermentation processes have not been disclosed for clavulanic acid manufacture.

According to the present invention a process for production of clavulanic acid comprises fermentation of a clavulanic acid producing species of Streptomyces in a fermentation broth containing assimilable sources of carbon and nitrogen, wherein the concentration of phosphorus in the fermentation broth is less than 0.15% w/v.

The phosphorus concentration is preferably maintained below a limit of 0.15% w/v during the growth phase after which the phosphorus concentration may be allowed to decrease. The growth phase for a typical clavulanic acid fermentation lasting for a total of 5 to 6 days may be complete by about 40 hours. The source of assimilable phosphorus may be present as a phosphate salt for example sodium or potassium phosphate, sodium or potassium dihydrogen phosphate or disodium or dipotassium hydrogen phosphate or mixtures thereof. The phosphorus concentration referred to in this specification is determined as the percentage w/v of phosphorus equivalent to

the amount of assimilable phosphorus compound present.

The phosphorus concentration is preferably 0.0015 to 0.15% w/v, more preferably 0.002 to 0.015% w/v. The phosphorus concentration is preferably allowed to reduce to a low value, preferably zero by the 40th hour of fermentation.

Regulation of the amount of assimilable phosphorus in accordance with the present invention may afford unexpectedly high yields of clavulanic acid.

The concentration of assimilable carbon source may be selected by routine trials dependent on the characteristics of the Streptomyces strain employed. The proportion of a carbon source such as glycerol, glycerol trioleate or corn starch in the starting medium may be higher than 5% w/v and further quantities of a carbon source may be added during the fermentation in accordance with usual fed batch procedures.

Assimilable nitrogen may be provided by proteinaceous matter in the starting media. Alternatively or in addition ammonia may be introduced into the fermenter. Ammonia has also been used to regulate the pH of the fermentation broth during the course of the fermentation. However we have found that use of ammonia both as the sole source of assimilable nitrogen and also for pH regulation is undesirable. A high concentration of ammonia can poison the microorganisms and a pH which is too low results in less effective clavulanic acid biosynthesis. Accordingly it is preferred that an assimilable source of nitrogen, for example soya bean flour, is added to the starting medium and that an ammonium salt such as ammonium sulphate is added during the course of the fermentation to provide further nitrogen as necessary.

According to a preferred aspect of the present invention a nitrogen free compound, preferably ammonium hydroxide is used to control pH. This results in a later decrease in the level of biomass than is the case if ammonia is used as the sole nitrogen source and pH regulator. The results of a comparison with a classic fermentation are shown in Figure 1. The viscosity, which is proportional to the amount of the biomass is shown for a classical fermentation (broken line) and for a

fermentation in accordance with the invention (solid line).

The assimilable nitrogen source may be flour, for example soya flour or cotton seed flour. The amount of assimilable nitrogen is preferably 0.5 to 15% w/v, more preferably 1.5 to 7.5% w/v. The amount of nitrogen is advantageously greater than 5%.

The invention relates particularly to fermentation of the Streptomyces species S.clavuligerus NRRL 3585, S.jumonjinensis NRRL 5741 and S.katsurahamanus IFO 13716 and particularly Streptomyces sp.P6621 FERM P2804. The invention yield improved yields of clavulanic acid from S.clavuligerus The invention finds particular application in relation to commercial scale fermentations particularly but not exceeding broth volumes of 10^4 l, preferably 5 x 10^4 l

The fermentation broth may be treated as disclosed in our WO95/23870 or by other known methods by which potassium clavulanate of high purity may be prepared.

The invention is further described by means of example but not in any limitative sense.

EXAMPLE 1

A) CULTIVATION OF STREPTOMYCES SP.P 6621 FERM P 2804

Strain selection and maintenance

The most productive clones of Streptomyces sp. PP 6621 FERM P 2804 were obtained by selection methods. The most productive cultures of this microorganism were stored and were further used as a source for new selection cycles.

A colony of Streptomyces sp. PP 6621 FERM P 2804 was aseptically transferred in to a sterile potter with sterile water ($2cm^3$) and homogenised. Fragments of the mycelium were transferred onto an agar slope and incubated to maturity (for 10 to 14 days) in a thermostat at 25°C.

After 8 to 10 days the agar surface was overgrown by a grey-green bacterial mycelium. Spores were scraped from the surface, aseptically inoculated into a seed vegetative medium and incubated on a shaker for 24 h at 250 rpm and at 25°±1°C.

Homogeneous suspension of spores from agar slopes may be stored in skimmed milk (which can be used as a protective medium) for more than two months.

After completion of the vegetative stage, part of the culture was aseptically transferred to a fermentation medium and was incubated on a rotary shaker for 96 h. After the finished fermentation state the content of clavulanic acid was analysed. Cultures which gave the highest yields were used as laboratory inoculum in the fermenter.

The entire procedure was carried out under aseptic conditions.

Strains may be stored on slope agar at 4°C maximum for 4 weeks, in skimmed milk in the same condition for 2 months and lyophilised strains may be stored at 4°C for a period of years.

Composition of media for selection of strain for inoculation in the fermenter

Media for slopes and Petri dishes

Composition	amount
dextrin	10 g
KH_2PO_4	1 g
$MgSO_4$ $7H_2O$	1 g
NaCl	1 g
$(NH_4)_2SO_4$	1 g
$CaCO_3$	4 g
Trace elements *	1 cm^3
agar	20 g
demineralised water	to 1000 cm^3

The composition was prepared in accordance with classical methods.

Trace elements*

Composition	amounts
$CaCl_2$ $2H_2O$	10.0 g
$MgCl_2$ $6H_2O$	10.0 g
NaCl	10.0 g
$FeCl_3$ $6H_2O$	3.0 g
$ZnCl_2$	0.5 g
$CuCl_2$ $2H_2O$	0.5 g
$MnSO_4$ H_2O	0.5 g
demineralised water	to 1000 cm^3

Vegetative media for strain selection

Composition	amounts
corn starch	10.0 g
soybean flour	20.0 g
KH_2PO_4	0.6 g
Estol (Priolube 1435)	5.0 g
tap water	to 1000 cm^3

Fermentation media for strain selection

Composition	amounts
corn starch	9.6 g
soybean flour	38.5 g
KH_2PO_4	1.2 g
Estol (Priolube 1435)	23.0 g
glycerol	5.0 g
morpholine propane sulphonic acid	12.0 g
trace elements*	10.0 ml
tap water	to 1000 ml

Preparation of the laboratory inoculum

The origin of culture for preparation of laboratory inocula was cultured from an agar slope. The chosen slope agar was filled aseptically with sterile water ($10cm^3$), spores were scraped off and homogenised in a sterile potter. The solution of spores was used as a laboratory inoculum.

VEGETATIVE PHASE IN PRE-SEED TANK

Media for pre-seed tank

Volume of pre-seed = 500l

Volume of medium = 350l

Composition	amounts
corn starch	7.0 kg
soybean flour	7.0 g
NaH_2PO_4	0.185 kg
Estol (Priolube 1435)*	0.7 kg
synperonic	0.150 kg
tap water	to 350 l

*soybean oil can be used instead of estol.

The inoculum was transferred in a medium that had been sterilised in pre-seed tank and cooled by sterile air to 28°C. The vegetative phase lasted from 50 to 70 hours at a temperature 28°±1°C, pressure 0.3 Bar and with aeration using sterile air and with consistent mixtures.

Parameters of growth in pre-seed tank

time/h (h)	pH	PMV%	decolourisation/min
0	7.20	--	--
4	7.25	10	> 5
10	7.35	8	> 5
16	7.30	10	> 5
22	7.20	16	4
28	7.02	17	2.5
34	6.85	18	0.5
39	6.66	20	0.3
45	6.60	21	0.5
51	6.52	22	1.0
56	6.39	22	1.0
61	6.45	20	1.3

Legend:
pH = pH value of sample
PMV% = volume % of culture in sample
decolourisation = time necessary for decolourisation of methylene dye

VEGETATIVE PHASE IN SEED FERMENTER

Media for seed fermenter
vol. of seed fermenter = 7500 l
vol. of media = 4500 l

Composition	amount
corn starch	90 kg
soybean flour	90 kg
NaH_2PO_4	2.4 kg
Estol (Priolube 1435)*	9 kg
Synperonic	0.5 kg
water	to 4500 l

* Soybean oil can be used instead of estol.

The vegetative phase from the pre-seed tank was transferred under pressure into a medium that had been sterilised in the seed fermenter and cooled by sterile air to 28°C. Air was sterilised by filters with a pore size of 0.2 µm.

The vegetative phase lasted from 10 to 20 h at 28°±1°C, pressure 0.3 Bar, aeration by sterile air and constant mixing.

Growth was monitoring by analysis of pH, PMV%, decolourisation of methylene and by microscopic examination of samples.

Parameters of growth in pre-seed tank

Time/h	pH	PMV%	declourisation/min
0	7.20	--	--
6	7.10	15	> 5
12	6.87	20	1.5
16	6.65	22	0.3

Legend:
pH = pH value of sample
PMV% = volume % of culture in sample
decolourisation = time necessary for decolourisation of methylene dye

B) BATCH FERMENTATION OF STREPTOMYCES SP. P 6621 FERM P 2804 IN FERMENTER

Media for fermenters
Vol. of fermenter = 90 000 l
Vol of media = 60 000 l

Composition	amount
corn starch	570 kg
soybean flour	2300 kg
NaCl	6 kg
Estol (Priolube 1435)*	1680 kg
NaH_2PO_4	5 kg
$MgCl_2$ $6H_2O$	7 kg
$FeCl_3$ $6H_2O$	1.6 kg
$ZnCl_2$	0.5 kg
$MnSO_4$ H_2O	0.1 kg
Synperonic	25 kg
water	to 60 m^3

Legend:
- Estol is a generic name for glycerol trioleate; (Priolube 1435 registered trade mark of Unichem GmbH, Germany)
- Synperonic (registered trade mark of ICI GB) is a propylenglycol antifoam agent
* Soybean oil can be used instead of Estol.

4700 l of a culture of Streptomyces sp. PP 6621 FERM P 2804 in the vegetative phase of growth from the seed fermenter was inoculated by a sterile transfer into a sterile starting medium (60 000l) in a 90 000 l stainless steel fermenter equipped for mixing and a delivery of sterile air through filters with a 0.2 μm pore size. The fermentation media and all inlet-pipes were sterilised and cooled by sterile air to 24°C. The fermentation phase from seed fermenter was maintained at 24°C - 25°C and 0.3 Bar. The broth was mixed and aerated during the course of whole fermentation and the pH of the media was maintained by addition of 25% aqueous solution of ammonium hydroxide at value 6.8 - 6.9. The fermentation lasted for 96h and the resultant concentration of clavulanic acid was 3580 mg/l.

During the course of the fermentation of Streptomyces sp.

PP 6621 FERM P 2804 a source of phosphorus and an assimilable source of nitrogen (500 kg of soybean flour in 5000 l of water and 25% aqueous solution of ammonium hydroxide) were added as follows:

Concentration of assimilable sources of phosphorus and nitrogen in the fermentation broth

time	phosphorus concentration (% w/v)	nitrogen concentrate (% w/v)
0	0,035	1,73975
8	0,030625	1,692286
16	0,0095	1,331
24	0.005188	0,9785
32	0,004638	0,5527
40	0,003638	0,69945
48	0,000863	0,9128
56	0,000863	0,8475
64	0	0,709
72	0	0,653625
80	0	0,571
88	0	0,47675
96	0	0,53825
104	0	0,7555
112	0	0,77025
120	0	0,673375
128	0	0,78725
136	0	0,734625
144	0	0,8985

The concentration of phosphorus after the 51st hour of the fermentation was below the detection limit.

The pH value reached in the first hours of the culture growth rose to almost 7.5. During this time phosphorus was consumed and clavulanic acid started to be produced, because of this the pH decreased and control of the pH of the media was

necessary to maintain the level of pH at the optimum value.

EXAMPLE 2

PROLONGATION OF THE VEGETATIVE PHASE OF FERMENTATION BY USE OF AMMONIUM SULPHATE AS ASSIMILABLE SOURCE OF NITROGEN AND SODIUM HYDROXIDE AS REGULATOR OF PH

A medium used with the same proportions of ingredients as Example 1B was placed in two stainless steel fermenters (500l each). The fermentation in the first fermenter was run under the same conditions as were described in the Example 1B. The fermentation conditions in second fermenter differed from that described in Example 1B only in that an 11% aqueous solution of ammonium sulphate at 9 cm^3/l was added to the fermentation broth during the period between the 40th and 60th hours following inoculation. The pH was maintained on the desired level by sodium hydroxide. After the 60th hour we stopped the addition of nitrogen source was stopped. The viscosity of the fermentation broth, which is proportional to the amount of biomass, was analysed during the course of fermentation.

Time	Run 1, viscosity (m Pa.s)	Run 2, viscosity (m Pa.s)
0	/	/
8	/	/
26	474	551
44	728	714
62	948	998
80	995	1076
98	936	1226
116	824	863
128	628	873